SELECTING AND STABILIZING DSRNA CONSTRUCTS

[0001] This application claims priority to U.S. Provisional Patent Application 60/772,736, filed Feb. 13, 2006, which is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] The present invention relates to stable expression of RNAi constructs in plants to enable genetic control of plant pathogens and pests. The invention provides methods and compositions for improving the efficacy of dsRNAs derived from such constructs.

[0004] 2. Description of Related Art

[0005] Short strands of complementary double stranded RNA (dsRNA) when present in, or introduced into, living cells may specifically affect the expression of a "target" gene when regions of nucleotide sequence similarity are shared between the dsRNA and the target gene transcript. Such RNA molecules may comprise complementary sequences separated by a "spacer" region such that double stranded regions of RNA are formed. The dsRNA may be cleaved by enzymes known as dimeric RNase III ribonucleases (also called "dicer" enzymes) into segments approximately 21-25 base pairs in length; called siRNAs ("short interfering RNAs" or "small interfering RNAs"). The siRNA causes specific RNAse activity in a RNA-induced silencing complex ("RISC") to hydrolyze the target gene mRNA, thereby post-transcriptionally suppressing expression of the target gene. Only transcripts complementary to the siRNA are cleaved and degraded, and thus the effect, sometimes called RNA interference (RNAi), is gene specific. RNAi has been used to specifically disrupt gene expression in a number of organisms including *Caenorhabditis elegans* (Fire et al., 1998), *Drosophila melanogaster*, insects including *Coleoptera* (Bucher et al., 2002) and *Lepidoptera* (Uhlirova et al. 2003; Bettencourt et al., 2002), fungi (Cogoni et al. 2000), and plants such as *Arabidopsis thaliana*, among others. dsRNA present in plants may also guide DNA methylation of targeted chromatin regions, resulting in gene silencing (e.g. Wassenegger et al., 1994; Carthew, 2001; Zilberman et al., 2004).

[0006] Effective use of RNAi leads to suppression of expression of a specific target gene, and thus stable expression of RNAi constructs in transgenic crops can allow for novel genetic approaches to pest control. However dsRNA produced from a transgene in planta, although targeted to another organism, may evoke in planta responses such as cleavage ("dicing") of a transgene transcript, as well as silencing of the cognate transgene in the transgenic host plant. These responses could reduce or eliminate dsRNA production and hence efficacy against a target organism.

[0007] There have been reports concerning design of constructs for evoking dsRNA-mediated suppression of gene expression (Wesley et al., 2001; Yuan et al., 2004; Reynolds et al., 2004; Arziman et al., 2005). Mechanisms for systemic transport of sRNA ("small RNA") molecules (including dsRNA) are known in some organisms (e.g. Voinnet 2005), and the sequence of the ribonucleotide being transported is known to have an effect on the efficiency of its uptake (Winston et al., 2002). For instance, *C. elegans* requires a dsRNA of roughly 100 base pairs (bp) in length to be productively taken up into gut cells e.g. via SID1 protein (Feinberg and Hunter, 2003), and WO9953050 describes dsRNA constructs comprising intron sequences in spacer regions. However the parameters leading to optimized production, stabilization, and uptake of dsRNA active against a target pest, while ensuring stable expression of a transgene encoding such dsRNA, and avoiding transgene silencing in a host cell, are not well understood. Thus there exists a need to ensure stable transcription of specific effective dsRNA-encoding transgenes within plants, and subsequent transport and uptake of the resulting dsRNA, to yield effective and specific gene suppression in target plant pathogen and pest species.

BRIEF DESCRIPTION OF THE FIGURES

[0008] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0009] FIG. **1**A-**1**B: Alignment of a 100 bp segment of the Dv49 target with related sequences from other organisms representing multiple genera, orders and phyla. Sequences differing from *Diabrotica virgifera virgifera* (Dv49) are highlighted. Amino acid alignment (a.a.) for the Dv49 conceptual translation is shown below the nucleotide sequence. Reynolds scores were calculated for the Dv49 sequence and are shown below the amino acid alignment—the score position corresponds to nucleotide 19 of the antisense strand 21mer. Data from the embedded 26mer efficacy scan are presented below the Reynolds score. The potential 21mers that could be produced from each scan segment are underlined and the WCR mortality resulting from each embedded segment fed at 0.2 ppm in artificial diet bio-assay is shown below each scan segment. * significantly different from untreated control, P value <0.05, Planned Contrasts.

[0010] FIG. **2**: Segments of coding sequence from a Na/K-exchanging ATPase (putative *Drosophila* gene, CG9261, ortholog) aligned from multiple *Diabrotica* spp. Sequence conforming to the group consensus is boxed and shaded. Sequencing has shown presence of alleles in some instances (e.g. "R" at position 49 of NCR sequence).

[0011] FIG. **3**: Phylogenetic tree determined using a 559 bp segment of Dv26 and the ClustalW algorithm in the DNASTAR software package (Madison, Wis.).

[0012] FIG. **4**: Design for transgene that reduces direct contiguous sequence identity between transcript of gene and resulting dsRNA transcript. Transcription unit could be terminated by a synthetic sequence derived from siRNAs that are not productively incorporated into RISC.

[0013] FIG. **5**: Small efficacious dsRNA segments for insertion into expression cassette at indicated sites.

[0014] FIG. **6**: 300 bp segments of *Diabrotica virgifera* V-ATPase subunit A for assay as dsRNA in WCR diet bioassay. UTC=untreated control. EST=a short V-ATPase subunit A cDNA clone that lacked sections 1 and 2.

[0015] FIG. **7**: Dv49 embedded approx. 26mer efficacy scan fed at 1 ppm.

[0016] FIG. **8**: Dv49 embedded approx. 26mer efficacy scan fed at 0.2 ppm.

[0017] FIG. **9**: Dv49 scan 14 27mer segment scanned as 21mers and tested for efficacy at 0.2 ppm.